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Thermal unfolding of monomeric Ca(II),Mg(II)-ATPase from sarcoplasmic reticulum of rabbit skeletal muscle

Jaime M. Merino[a], Jesper V. Moller[b], Carlos Gutiérrez-Merino[a,*]

[a]*Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Extremadura, 06080-Badajoz, Spain*
[b]*Institute de Biophysics, University of Aarhus, DK-8000 Aarhus C, Danemark*

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Abstract

The thermal unfolding of monomeric and delipidated Ca^{2+}-ATPase, solubilized in $C_{12}E_8$, can be appropriately described as a non-two-state irreversible denaturation, with only one endothermic peak. In the Ca^{2+} concentration range (0–0.5 mM) which stimulates the ATPase activity of solubilized monomeric ATPase, Ca^{2+} shifts the critical temperature midpoint of the denaturation process (T_m) from 42 to 50°C without segregation of the endothermic peak into two separate components. Because 20 mM Mg^{2+} only shifts the T_m from 42 to 44°C, we conclude that the effect of Ca^{2+} upon the T_m is likely to be due to binding to the high affinity Ca^{2+} sites in the ATPase. The effect of Ca^{2+} upon the enthalpy of denaturation is biphasic, suggesting the presence of low affinity Ca^{2+} sites ($K_{0.5}$ in the millimolar range) in monomeric and solubilized ATPase.

Key words: Ca^{2+},Mg^{2+}-ATPase; Sarcoplasmic reticulum; Monomeric ATPase, Differential scanning calorimetry; Ca^{2+}; Mg^{2+}-ADP

1. Introduction

The thermal unfolding of the Ca^{2+},Mg^{2+}-ATPase (ATPase) in sarcoplasmic reticulum (SR) membranes has been shown to take place between 47 and 55°C [1–3]. The overall shape of the thermograms shows that with SR membranes purified according to current protocols, unfolding of the ATPase is a complex process, showing an exothermic peak at slightly higher temperatures than the major part of the unfolding denaturation peak, which prohibits the fine thermodynamic analysis of the unfolding beyond highly restrictive experimental conditions [1]. Based on their analysis of the thermograms of SR membranes, Lepock et al. [2] have suggested that two distinct functional domains of the ATPase unfold separately, one of which corresponds to that which binds Ca^{2+} (presumably containing the Ca^{2+} transport sites) and the other one the catalytic center. From a structural viewpoint this implies a large degree of freedom of the Ca^{2+} binding domain with respect to the catalytic domain, a point which is of particular relevance to the molecular mechanism of coupling between the ATPase activity and Ca^{2+} transport. However, SR membranes are contaminated with glycogen phosphorylase [4–7] which unfolds in a temperature range close to that of the ATPase, namely 49–65°C [8], and in the presence of glycogen phosphorylase the unfolding of SR membranes shows a complex pattern with a large endothermic peak [8].

Using $C_{12}E_8$, ATPase can be solubilized from the SR membrane in a monomeric and delipidated form with retention of full catalytic activity for hours at 25°C [9], provided that solubilization is performed under appropriate experimental conditions. Monomeric ATPase has been shown to be the minimum catalytic unit of the ATPase [10]. Ca^{2+} and, to a lesser extent, Mg^{2+}-ADP have been reported to stabilize solubilized monomeric ATPase [9]. Therefore, a priori such preparations of monomeric ATPase may be suited for the study of the basic thermodynamic properties of thermal unfolding of the ATPase and to overcome the drawbacks mentioned above.

2. Materials and methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle (New Zealand white) as in [11,12]. Ca^{2+}-ATPase was purified by extraction from SR membranes with a low concentration of deoxycholate according to [13]. SR membranes were treated to remove glycogen phosphorylase as indicated in [7]. Monomeric and delipidated Ca^{2+}-ATPase (less than 1 mol lipid per mol of protein) was prepared as previously described [14], and cryo-preserved by quick freezing after addition of 20% (v/v) glycerol. Protein concentration was measured following the method of Lowry using bovine serum albumin as a stand-

*Corresponding author. Fax: (34) (24) 27 13 04.

Abbreviations: SR, sarcoplasmic reticulum; ATPase, Ca(II) + Mg(II)-ATPase (EC 3.6.1.38); $C_{12}E_8$, octaethylene glycol dodecyl ether; I.U., micromoles of product per minute per milligram of protein; DSC, differential scanning calorimetry; ΔH, enthalpy of denaturation process; T_m, critical temperature midpoint of denaturation process.

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ard [15]. Phospholipids were determined from lipid phosphorous as in [16].

Ca^{2+}-ATPase activity was measured spectrophotometrically using the coupled enzyme pyruvate kinase/lactate dehydrogenase assay [17] with the following reaction mixture: 0.01 M TES/KOH (pH 7.5), 0.1 M KCl, 0.1 mM $CaCl_2$, 6 mM $MgCl_2$, 5 mM ATP, 0.82 mM phosphoenolpyruvate, 0.16 mM NADH, 18 I.U. lactate dehydrogenase and 18 I.U. pyruvate kinase. To measure the activity of the monomeric ATPase, 1 mg/ml C_{12}/E_8 was added to the reaction medium. The activity of monomeric Ca^{2+}-ATPase used in this study ranged from 6 to 7 I.U. at 25°C, and showed negligible Ca^{2+}-independent ATPase activity as indicated by its complete inhibition upon addition of 3 mM EGTA.

Scanning calorimetry measurements were carried out, using a differential scanning calorimeter MicroCal MC-2, operated at a scanning rate of 30 or 60°C/h, and under a nitrogen pressure of 1.5–2 kg/cm^2 during the scan. All the scans reported in this paper are representative of, at least, triplicate experiments carried out with triplicate preparations of monomeric ATPase or SR membranes. The concentration of protein was always between 1.5 and 2 mg/ml. The buffer used in DSC experiments was 20% glycerol, 0.1 M KCl, 0.02 M TES/KOH (pH 7.0). Ca^{2+} and EGTA were added as required. The samples were degassed before loading the calorimeter. The analysis of the calorimetric data was carried out with the Origin TM software developed by MicroCal, Inc. for the MC-2 differential scanning calorimeter.

The computation of free Ca^{2+} concentrations was made using the computer program described by Perrin and Sayce [18], with the following K_d values at pH 7.0 [19]: 1.55 mM (Ca^{2+}-ADP), 1 mM (Mg^{2+}-ADP), 0.4 μM (Ca^{2+}-EGTA) and 25.1 mM (Mg^{2+}-EGTA).

3. Results and discussion

To stabilize solubilized, monomeric ATPase during a time period long enough for sample handling and thermal pre-equilibration of the DSC microcalorimeter (currently 45 min) 20% glycerol and 0.5 mM $CaCl_2$ were added to the buffer as indicated in [9]. Before every DSC run an aliquot of frozen monomeric ATPase was thawed and transiently kept on ice. After freezing and thawing the aggregation state of the ATPase was checked by HPLC on TSK G3000SW of 7.5 × 600 mm columns [14] and found to elute as a monomer peak with no indications of reaggregation (results not shown). In the titrations with Ca^{2+}, to obtain concentrations of free Ca^{2+} lower than 0.5 mM, appropriate EGTA concentrations were added together with 3 mM Mg^{2+}-ADP (to avoid significant loss of activity by the Ca^{2+}-depleted enzyme, [9]. Under these experimental conditions, ATPase loses activity at a rate lower than 1% per hour at 25°C, e.g. less than 10% loss of activity after 10 h (results not shown). Fig. 1 shows typical thermograms of the unfolding of monomeric ATPase in the presence of 0.1 μM (trace a) and of 0.5 mM Ca^{2+} (trace b). The overall thermal unfolding of the monomeric ATPase is an irreversible process as illustrated by trace f of Fig. 1, and can be satisfactorily fitted only with the algorithm of a non-two-state transition. T_m is not very sensitive to changes of the scanning rate from 30 to 60°C/h (compare traces b and c of Fig. 1). In contrast to earlier results reported with SR membranes [1,2,8] only one major endothermic peak is evident in the thermograms shown in Fig. 1, with a small shoulder approximately 5°C above the T_m in the case of SR membranes of low glycogen phosphorylase

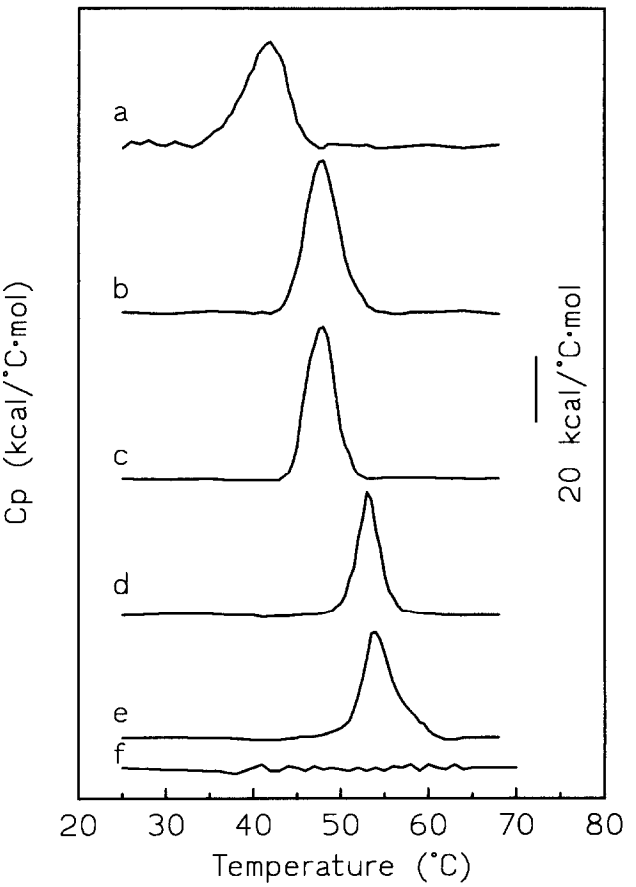


Fig. 1. DSC thermograms of monomeric Ca^{2+}-ATPase (1.25 mg/ml) solubilized in $C_{12}E_8$ (1 mg/ml) and of SR membranes (2.0 mg/ml). Scanning rate: 30°C/h (traces a, b, d and f) and 60°C/h (traces c and e). Thermograms of monomeric Ca^{2+}-ATPase in the presence of 0.5 mM $CaCl_2$ plus 2 mM EGTA and 3 mM Mg^{2+}-ADP (trace a), or in the presence of 0.5 mM $CaCl_2$ (traces b and c). Trace f, re-scan of the sample corresponding to the thermogram of trace a after heating up to 75°C. Traces d and e, thermograms of SR membranes (2.0 mg/ml) in the presence of 0.5 mM $CaCl_2$. Buffer: 20% v/v glycerol/0.1 M KCl/20 mM TES/KOH (pH 7.0).

content (trace d). Because the enthalpy of this shoulder varies slightly (from 2 to 6% of the total enthalpy of denaturation) from preparation to preparation of SR membranes treated to remove glycogen phosphorylase, as indicated in [7], and even it is negligible in many SR preparations, it is likely that it is reflecting the contribution of residual glycogen phosphorylase in SR membranes. In addition with SR membranes of low glycogen phosphorylase contamination, increasing the scanning rate from 30 to 60°C/h (to approach further to the experimental conditions used in [1,2]) only produces a slight increase of the T_m and a more skewed endothermic peak (traces d and e of Fig. 1), as expected for an unfolding process consisting of, at least, one reversible step before the irreversible transition, but not a clear segregation of the endothermic peak into two distinct peaks.

To confirm that the endothermic peak of DSC scans

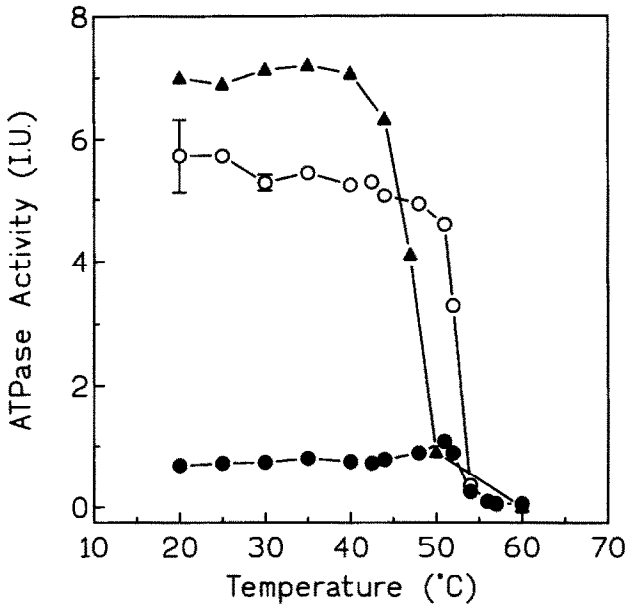


Fig. 2. Thermal inactivation of SR Ca^{2+}-ATPase. Samples of monomeric Ca^{2+}-ATPase (1.25 mg/ml) (▲) in 20% v/v glycerol/0.5 mM $CaCl_2$/0.1 M KCl/20 mM TES/KOH (pH 7.0) plus $C_{12}E_8$ (1 mg/ml) or of SR membranes (2 mg/ml) (○, ●) in 20% v/v glycerol/0.1 M KCl/20 mM TES/KOH (pH 7) were placed in a programmable Haake water bath and heated at a rate of 30°C/h, to simulate the experimental conditions of DSC runs. At different temperatures aliquots were pooled and assayed for Ca^{2+}-dependent ATPase activity at 25°C as indicated in section 2, in the presence (▲, ○) or in the absence of 1 mg/ml $C_{12}E_8$ (●) in the assay medium. Samples heated at 75°C were cooled down to 25°C, and were found to have irreversibly lost the ATPase activity.

of monomeric ATPase and of SR membranes is due to denaturation of the Ca^{2+}-ATPase we have measured the dependence upon temperature of this activity, with samples continuously heated at a rate of 30°C/h using a programmable water bath and in the buffer used in DSC runs (Fig. 2). The results show that the major endothermic peak overlaps with the temperature range at which the Ca^{2+}-ATPase activity is irreversibly inactivated.

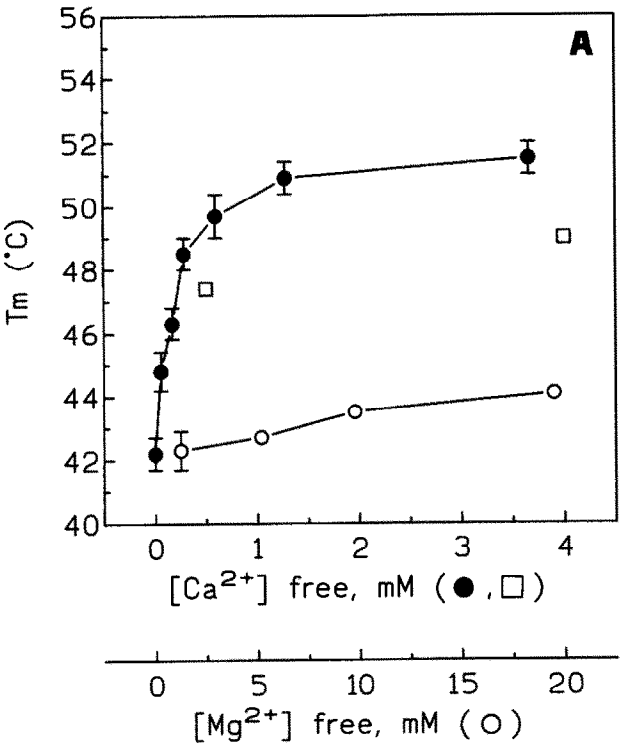
The results of the analysis of the thermograms of monomeric ATPase and of SR membranes run under the experimental conditions of Fig. 1 are presented in Table 1. The ΔH values obtained in this study for SR membranes are about 10–20% lower than those reported previously [1,2]. This cannot be attributed to significant denaturation of the ATPase during the treatment of SR membranes to remove glycogen phosphorylase and other non-transmembranal proteins associated to SR membranes, because this treatment does not produce a significant loss of the specific Ca^{2+}-ATPase activity [7], and results not shown. Consistently, DSC scans run with a buffer minus glycerol yielded ΔH values similar to those reported previously using SR membranes not treated to remove associated proteins [1,2]. It can be seen that solubilized, monomeric ATPase in the presence of 0.1 μM Ca^{2+} and 3 mM Mg^{2+}-ADP is more than 10°C more unstable than the ATPase in SR membranes. However, in the presence of Ca^{2+} alone at a concentration of 0.5 mM, monomeric ATPase is further stabilized to produce an increase of approx. 5°C of the T_m of the endothermic peak. We wondered whether this partial stabilization afforded by Ca^{2+} could reflect specific binding of Ca^{2+} to the translocation sites of monomeric ATPase. Therefore, we carried out titrations with increasing Ca^{2+} concentrations in the buffer. Fig. 3A shows that T_m for detergent solubilized Ca^{2+}-ATPase is steeply rising as a function of free Ca^{2+} concentration below 0.5 mM and only moderately above this level. At 0.5–4 mM free Ca^{2+}, withdrawal of Mg^{2+}-ADP from the medium only reduces T_m by about 2°C (Fig. 3A). This is thus the stabilization afforded by the Mg^{2+}-nucleotide complex at saturation of the enzyme by Ca^{2+}. Titrations with Mg^{2+} up to 20 mM produced at most an increase of 2°C of the T_m of monomeric ATPase (Fig. 3A), hence supporting that the effect of low Ca^{2+} is not unspecific, but due to binding to well defined Ca^{2+} sites in the monomeric ATPase. However, a biphasic effect of Ca^{2+} concentration is clearly observed on ΔH values of monomeric and solubilized ATPase (Fig. 3B). Interestingly, Mg^{2+} (5–20 mM) also produces a decrease of ΔH, similar to the decline seen at high Ca^{2+} concentrations (results not shown). Therefore, it seems likely that the effects of millimolar Ca^{2+} concentration upon the thermal denaturation of solubilized monomeric ATPase are due to binding to sites in the ATPase with low specificity for divalent cations.

From Fig. 3A a $K_{0.5}$ value of 150 ± 10 μM Ca^{2+} can be obtained for Ca^{2+} binding to centers that stabilize monomeric ATPase against thermal denaturation. This $K_{0.5}$ value is quite close to the $K_{0.5}$ value (40 μM) of activation to Ca^{2+} of the solubilized monomeric ATPase under the same experimental conditions (e.g. in the presence of 1 mg/ml $C_{12}E_8$ and 20% v/v glycerol), and it is to be noted that the maximum activity of the bell-shaped curve of dependence of the solubilized monomeric ATPase activity upon free Ca^{2+} concentration (Fig. 4) is attained at approx. 0.5 mM, a Ca^{2+} concentration that produces more than 90% of the change of T_m afforded

Table 1
Thermodynamic properties of the thermal unfolding of Ca^{2+}-ATPase

	T_m (°C)	ΔH (kcal/mol)
Monomeric ATPase[1]		
+ 2 mM EGTA/3 mM Mg^{2+}-ADP	42.3 ± 0.6	300 ± 60
+ 0.5 mM Ca^{2+}	47.4 ± 0.7	386 ± 75
SR membranes		
+ 2 mM EGTA	52.6 ± 0.1	279 ± 31
+ 2 mM EGTA/3 mM Mg^{2+}-ADP	54.5 ± 0.4	280 ± 37
+ 0.5 mM Ca^{2+}	53.5 ± 0.5	210 ± 33

Buffer: 20% glycerol, 100 mM KCl and 20 mM TES/KOH (pH 7.0).
[1]Solubilized in $C_{12}E_8$ (1 mg/ml).



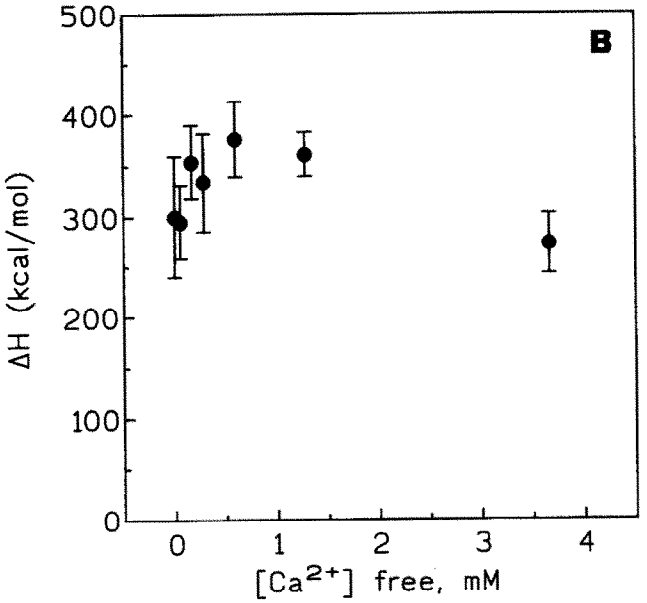


Fig. 3. Panel A: dependence upon free Ca^{2+} and Mg^{2+} concentration of the peak temperature (T_m) of the endothermic transition of the thermal unfolding of monomeric Ca^{2+}-ATPase solubilized in $C_{12}E_8$ (1 mg/ml). Scanning rate: 30°C/h. Other experimental conditions: (●) 20% v/v glycerol/0.1 M KCL/20 mM TES/KOH (pH 7.0)/0.5 mM $CaCl_2$/3 mM Mg^{2+}-ADP, EGTA and $CaCl_2$ were added as needed; (□) 20% v/v glycerol/0.1 M KCl/20 mM TES/KOH (pH 7.0)/0.5 mM $CaCl_2$ or 4 mM $CaCl_2$; (○) 20% v/v glycerol/0.1 M KCl/20 mM TES/KOH (pH 7.0)/0.5 mM $CaCl_2$/2 mM EGTA/3 mM Mg^{2+}-ADP, $MgCl_2$ was added as needed. Panel B: dependence upon free Ca^{2+} concentration of the ΔH (●) value of the thermal unfolding of monomeric Ca^{2+}-ATPase solubilized in $C_{12}E_8$ (1 mg/ml) in the Ca^{2+}/EGTA buffer of Panel A.

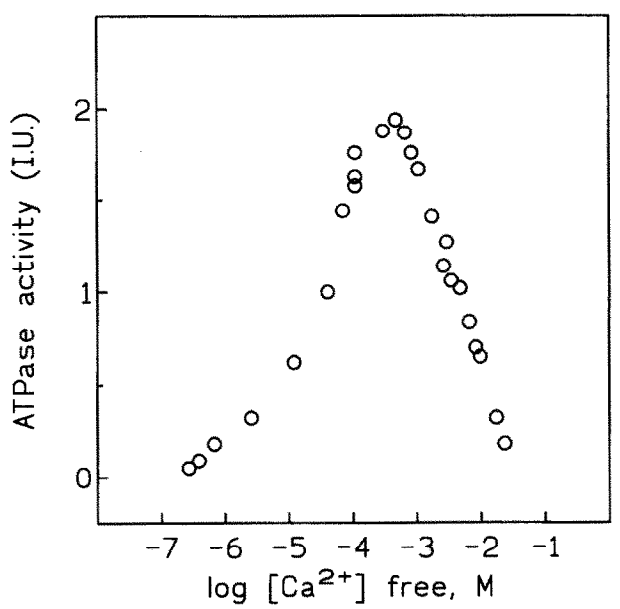


Fig. 4. Dependence upon free Ca^{2+} concentration of the ATPase activity of monomeric ATPase in the buffer indicated in Fig. 1. Other experimental conditions as indicated in section 2.

by Ca^{2+}. In addition, the $K_{0.5}$ value for inhibition of solubilized monomeric ATPase under these experimental conditions (Fig. 4) is approx. 4 mM, which could be due to binding of Ca^{2+} to the low affinity sites that produce lowering of ΔH in thermal denaturation.

The unstability of monomeric Ca^{2+}-ATPase in the absence of Ca^{2+} is the most striking difference between the thermal unfolding of solubilized and delipidated, monomeric Ca^{2+}-ATPase and that of Ca^{2+}-ATPase in SR membranes. The large protection afforded by Ca^{2+} against unfolding of monomeric Ca^{2+}-ATPase (ΔT_m of 10°C at saturation) contrasts with the very small effect that Ca^{2+} produces on SR membranes (ΔT_m < 1°C). This strongly suggests that it is the Ca^{2+} binding domain which is first unfolded in monomeric Ca^{2+}-ATPase, especially in the presence of Mg^{2+}-ADP. On the contrary, in SR membranes Mg^{2+}-ADP produces a stronger protection against thermal unfolding of the Ca^{2+}-ATPase than does Ca^{2+} (see Table 1). Therefore, in SR membranes the nucleotide binding domain that largely protrudes from the lipid bilayer seems to be unfolded first.

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